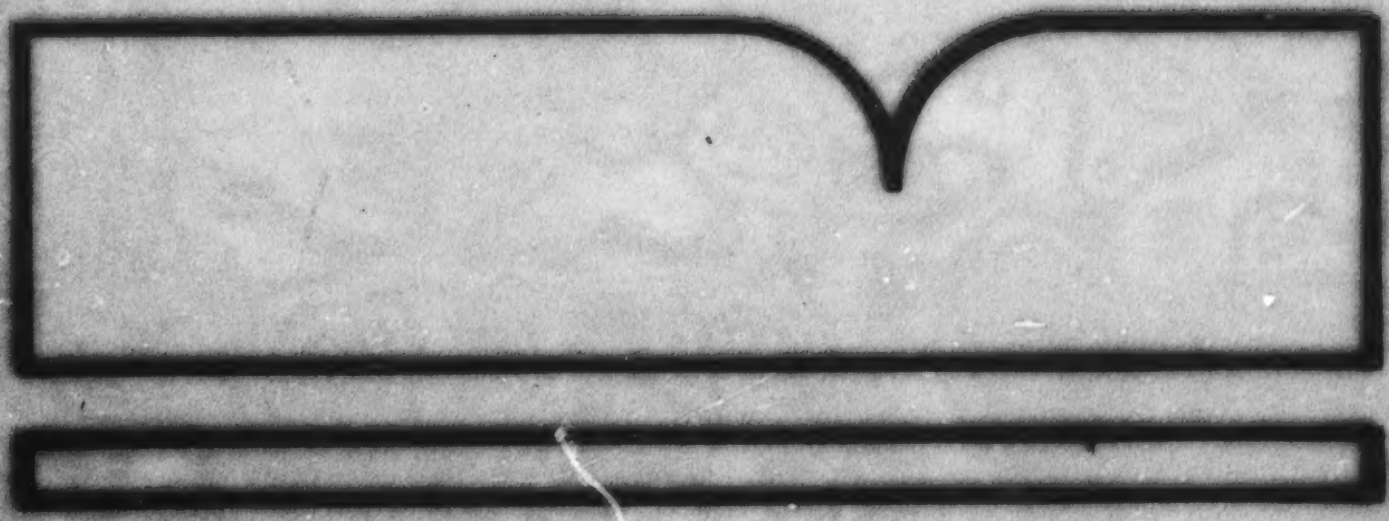
PB82-261397

Evaluation of 'Daphnia magna' Renewal Life-Cycle Test Method with Silver and Endosulfan

(U.S.) Corvallis Environmental Research Lab., OR

Jul 81



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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3.
EPA-600/J-82-220	JOURNAL ARTICLE	PB82-261397

4. TITLE AND SUBTITLE	5. REPORT DATE
Evaluation of a Daphnia magna Renewal Life-Cycle Test Method with Silver and Endosulfan	1982
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
A.V. Nebeker	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Environmental Research Laboratory Office of Research and Development U.S. Environmental Protection Agency Corvallis, Oregon 97333	10. PROGRAM ELEMENT NO.
	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
same	journal article - inhouse
	14. SPONSORING AGENCY CODE
	EPA/ORD/02

15. SUPPLEMENTARY NOTES

Published in Water Research Vol. 16, pp 739-744, 1982

16. ABSTRACT

Four contract and two U.S. Environmental Protection Agency laboratories participated in round-robin tests using Daphnia magna as the test animals. The purpose was to determine if methods set forth in the "Proposed Standard Practice for Conducting Renewal Life Cycle Tests with Daphnids" (ASTM Drafts 2 and 4 with modification) are adequate for routine laboratory testing. Silver and endosulfan were the toxicants used. The 48-h acute test used for determining EC50 values and estimating initial concentrations for the chronic test has been used for many years with good results, and this round robin confirmed its value. The renewal life-cycle test as proposed by American Society for Testing and Materials was found to be potentially useful, but only three of the six laboratories completed the tests successfully and they had many difficulties. Culture and feeding procedures and care of brood animals in cultures prior to testing were found to be weak links in the test procedure. If young animals used to start the test were not healthy, the test was not likely to succeed, primarily because of the problem of maintaining at least 80% survival of control animals. Excessive handling during transfer to new solutions directly damaged animals during pipetting and stressed brood-carrying adults. Feeding rates of 30 mg food l^{-1} test water depleted dissolved oxygen. Lower feeding rates and modification of rations were suggested. The preparation of fresh test solutions 3 times a week, with analyses to verify the concentrations is time consuming, expensive and presents many opportunities for error.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to public	unclassified	
	20. SECURITY CLASS *(This page)*	22. PRICE
	unclassified	

EPA-600/J-82-220
JOURNAL ARTICLE
0043-1354/82/050739-06$03.00/0

Water Res. Vol. 16, pp. 739 to 744, 1982
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EVALUATION OF A *DAPHNIA MAGNA* RENEWAL LIFE-CYCLE TEST METHOD WITH SILVER AND ENDOSULFAN

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(*Received July* 1981)

Abstract—Four contract and two U.S. Environmental Protection Agency laboratories participated in round-robin tests using *Daphnia magna* as the test animals. The purpose was to determine if methods set forth in the "Proposed Standard Practice for Conducting Renewal Life Cycle Tests with Daphnids" (ASTM Drafts 2 and 4 with modification) are adequate for routine laboratory testing. The six facilities were Haskell Laboratory, DuPont Co.; WAPORA, Inc.; Analytical Bio Chemistry Laboratories, Inc.; SRI International; Environmental Research Laboratory-Corvallis (EPA); and Environmental Research Laboratory-Duluth (EPA). Silver and endosulfan were the toxicants used.

The 48-h acute test used for determining EC50 values and estimating initial concentrations for the chronic test has been used for many years with good results, and this round-robin confirmed its value. The renewal life-cycle test as proposed by American Society for Testing and Materials (ASTM) was found to be potentially useful, but only three of the six laboratories completed the tests successfully, and they had many difficulties.

Forty-eight hour EC50 values (no food) for silver were 48, 55 (high values due to hard water), 8.4, 14.9, 1.1, 0.6, 0.64, 0.66, 0.9, 1.03, and 2.9 μg^{-1}. The 48-h EC50 values for endosulfan were 390, 630, 720, 372, 328, 343, 271, 218, 282, 266, 158, 280, 740 (LC50) and 378 (LC50) μg^{-1}. The 21-day EC50 values for silver were 2.9, 3.6 and 3.9; for endosulfan they were 170 and 130 μg^{-1}. Chronic MATC values based on reproduction for silver were 10.5–21.2, 20–41, 1.6–4.1, 8.8–19.4, 3.4–8.0 and 2.7–3.9 μg^{-1}. Chronic MATC values for endosulfan were 35–73, 75–154, 20–32 and 32–48 μg^{-1}.

Culture and feeding procedures and care of brood animals in cultures prior to testing were found to be weak links in the test procedure. If young animals used to start the test were not healthy, the test was not likely to succeed, primarily because of the problem of maintaining at least 80% survival of control animals. Excessive handling during transfer to new solutions (3 times a week for 4 weeks) directly damaged animals during pipetting and stressed brood-carrying adults. Feeding rates of 30 mg food l^{-1} test water depleted dissolved oxygen. Lower feeding rates and modification of rations, as by addition of vitamin enriched algae, were suggested. The preparation of fresh test solutions 3 times a week, with analyses to verify the concentrations, is time consuming, expensive, and presents many opportunities for error. The laboratories felt that the flow-through daphnid life-cycle test is more reliable for most test purposes and that EPA should recommend it in addition to the renewal test.

INTRODUCTION

The U.S. Environmental Protection Agency is required by the Toxic Substances Control Act of 1976 (P.L. 94-469) to standardize test procedures for evaluating the toxicity and persistence of new chemicals before they are approved for manufacture. The EPA Environmental Research Laboratories have been charged with responsibility for developing and validating toxicity tests using representative organisms from fresh water. The primary objective of this project was to evaluate a method developed by the American Society for Testing and Materials (ASTM), "Proposed Standard Practice for Conducting Renewal Life-Cycle Toxicity Tests with the daphnid, *Daphnia magna*", and to provide recommendations regarding its suitability as a standard procedure. This report summarizes the results generated from tests conducted by four private and two EPA laboratories (Table 1) to determine variability of test results collected by several laboratories following standardized procedures and the suitability of the method for EPA.

Methods development for *Daphnia* chronic toxicity tests has drawn on many sources, federal and state research laboratories, university research programs and private organizations. The U.S. Environmental Protection Agency (EPA) has been working with the American Society for Testing and Materials (ASTM) to coordinate the development of a standard practice for conducting renewal life cycle toxicity tests with the daphnid, *Daphnia magna*. Draft No. 2 of that method, prepared by Stephan (1977), forms the basis of the methods used in this project. Some modifications for this round-robin were necessary to accommodate the transition between Drafts Nos 2 and 4 (Comotto, 1979), and these are provided where appropriate. The method which takes into consideration results of this study is now in final development under the direction of Rita Comotto. Also in preparation (Adams, 1980) is an ASTM flow-through method formalizing

Table 1. Laboratory water quality: mean values during test period

Laboratory	pH	Alkalinity (mg l^{-1}) (as $CaCO_3$)	Hardness (mg l^{-1}) (as $CaCO_3$)
ABC*	7.8	368	255
DuPont†	8.6	84	73
ERL-Corvallis‡	7.2	49	60
ERL-Duluth§	7.7	40	46
SRI¶	7.4	50	46
WAPORA‖	7.0	32	54

* Analytical Bio Chemistry Laboratories, Inc., Columbia, MO 65205, U.S.A.
† Haskell Laboratory (E. I. DuPont Co.), Newark, DE 19711, U.S.A.
‡ Corvallis Environmental Research Laboratory, U.S. Environmental Protection Agency, Corvallis, OR 97333, U.S.A.
§ Environmental Research Laboratory, U.S. Environmental Protection Agency, Duluth, MN 55804, U.S.A.
¶ SRI International, Menlo Park, CA 94025, U.S.A.
‖ WAPORA, Inc., Berwyn, PA 19312, U.S.A.

methods that have been used by some laboratories for about ten years.

Two chemicals, endosulfan ($C_9H_6Cl_6O_3S$), a chlorinated cyclodiene insecticide registered under the trademark Thiodan® and silver nitrate ($AgNO_3$) were chosen for testing and sent to each laboratory. Endosulfan is used predominantly on fruit and vegetable crops: silver nitrate has several industrial and medical applications. They are representative of organic chemicals and heavy metals which occur in fresh waters of the United States and are very toxic to most aquatic organisms.

Papers by Adema (1978), Canton & Adema (1978), Biesinger & Christensen (1972), Maki (1979), Winner *et al.* (1977), Bertram & Hart (1979), Schwartz & Ballinger (1980) and Goulden & Hornig (1980) provide the most current information on culturing and testing of daphnids. Buikema *et al.* (1977) reviewed the literature on *Daphnia* toxicity tests and discussed procedures for using *Daphnia* in toxicity tests in the context of what is understood about its biology. Silver nitrate impact on *Daphnia* has been investigated and it appears to be one of the most toxic of the heavy metals. Mining drainage and wastes from photographic and electroplating industries are the principal sources of silver. Anderson (1948) reported that for *Daphnia magna* the toxic threshold concentration of silver was 3.2 $\mu g\,l^{-1}$. The U.S. Environmental Protection Agency (1978) reported a 48-h LC50 of 1.5 $\mu g\,l^{-1}$ in a static test with *Daphnia magna*. Bringman and Kühn (1977) reported a 24-h LC50 of 1.5 $\mu g\,l^{-1}$ for *Daphnia magna*. Nebeker *et al.* (1980) reported a 48-h EC50 of 0.9 $\mu g\,l^{-1}$ for *D. magna*. Endosulfan data are available for *Daphnia*. Nebeker *et al.* (1980) reported a *Daphnia magna* static 48-h EC50 value of 306 $\mu g\,l^{-1}$. *Daphnia magna* 48-h LC50 values of 166 and 132 $\mu g\,l^{-1}$ were reported by Macek *et al.* (1976) and Schoettger (1970), respectively. Macek (1976) reported an MATC for *Daphnia magna* of 4.3 $\mu g\,l^{-1}$ from a life cycle test.

METHODS AND MATERIALS

Acute tests

A 48-h EC50 was determined using ASTM standard practices for acute testing (ASTM, 1980), using 5 animals (<24 h old) per beaker, 4 beakers per concentration, with 5 concentrations plus controls. The EC50 is defined as that concentration where 50% of the animals are immobilized (lack of swimming or coordinated movement). The LC50 is used when death is verified by lack of movement and heartbeat. The EC50 was also used to determine the range of concentrations necessary for the renewal life-cycle test. 50% dilution between concentrations was used and 80% survival of controls or greater was considered acceptable. No food was given to the daphnids during the acute test.

Test temperatures were maintained at 20 ± 1°C, and summertime photoperiod (15–16 h light) was used. Test containers were 250 ml borosilicate glass beakers containing 200 ml of test solution. They were covered with glass plates to reduce evaporation of the test solution and prevent dust or other materials from contaminating the test. To eliminate agitation which would be harmful to the young daphnids, and to decrease losses of toxicant which might occur due to volatility, test beakers were not aerated during testing. Containers were cleaned with detergent and rinsed with water, and then rinsed with acetone, 5% nitric acid and distilled water as needed. They were rinsed with dilution water just prior to use.

Dilution water used for all testing was unchlorinated natural freshwater from wells or from surface sources. Water quality was variable, depending upon the laboratory and the time of year testing occurred (Table 1). Hardness, alkalinity, pH, dissolved oxygen and temperature were routinely monitored during testing. Measured toxicant values were used for data analysis.

Chronic tests

Separate groups of *Daphnia magna* were exposed to different concentrations of silver and endosulfan in test solutions. Each group was exposed for at least 21 days by transferring the organisms 3 times a week to a fresh solution containing the same concentration of toxicant. A control treatment of dilution water only provided a measure of test acceptability and served as a standard to evaluate effect concentrations. Results, calculated from data on the survival and reproduction of the daphnids, provided information about the effects of silver and endosulfan on test organisms under controlled laboratory conditions.

Atomic absorption spectroscopy was used to analyze silver and gas chromatography was used to measure endosulfan, using standard methodology. Acetone or triethylene glycol were used as the dispersant-solvents for endosulfan. All test concentrations were analyzed initially and during the course of the test, so that mean measured concentrations would be available for data analysis. The test solution was prepared in a flask with sufficient volume to fill all test containers, with enough remaining for analysis. During the test, concentrations were analyzed after the animals had been in the solution for 2 or 3 days, to determine how much toxicant was still in the test water.

The food used was PR-11 trout chow plus yeast (12 g PR-11 + 3 g yeast in 1 l. water), fed at a rate of 30 mg solids l^{-1}. PR-11 was formulated by Diet Development Center, Spearfish, South Dakota. Cultures were fed varying rates depending upon the laboratory, but test solutions were prepared with 30 mg solids l^{-1} of test solution.

Ten 250-ml beakers each containing 200 ml of the test solution were used for each concentration of silver and endosulfan. Each beaker contained one daphnid less than 24 h old at the start of the test, for collection of data on survival and reproduction. Five concentrations plus controls were set up for each test. Ten control beakers, consisting of the same dilution water, conditions, organisms, etc., but without toxicant, were used. Ten carrier (acetone or triethylene glycol) controls were also used with endosulfan to determine if the solvent had an impact on results.

Test solutions containing toxicant, food at 30 mg l^{-1} solids, and dilution water, were made up less than 4 h before starting the chronic test. For silver, the beakers were conditioned with test solution for 24 h prior to testing. This was not done with endosulfan. The test was started by placing one daphnid (< 24 h old) in each beaker containing 200 ml test solution. Each Monday, Wednesday and Friday, the live daphnid in each beaker was transferred to fresh toxicant solution at the same concentration as that from which it was removed. When young were present the parent daphnid was transferred to fresh solution and the young counted and discarded.

Adult survival and young per female per day were the parameters monitored during these tests. Number of young per female per day was calculated using number of young from day of first young until adult death or end of test.

The biological effects of silver and endosulfan on *Daphnia* during the life-cycle test were to be defined in terms of, first, an EC50 for parent survival after 21 days; this is defined as that concentration which immobilized 50% of the adults after 21 days. Second, toxicant impact on reproduction was defined as those concentrations which significantly ($\alpha = 0.05$) reduced the total number of young per female per day. Traditionally the observed effect concentration (on survival, growth and reproduction) and no observed effect concentrations are expressed as the MATC (Maximum Acceptable Toxicant Concentration) or NOEC or NOEL (No Observed Effect Concentration or Level). The lowest concentration which shows an adverse significant difference from the control is defined as the observed effect level or concentration (OEC). The next test concentration lower than the observed effect level is by definition termed the no effect level or concentration.

The 48-h, 21-day, and 28-day EC50 values were obtained using logistic regression methods (Finney, 1977). The reproductive data from Corvallis and DuPont were analyzed by analysis of variance and Dunnett's multiple comparison test (Steel & Torrie, 1960). Duluth used linear regression to analyze the number of young per female per day and the two test concentrations bracketing that concentration causing 50% reduction of young per female per day were taken as the MATC limits.

RESULTS

Acute tests

The 48-h EC50 values generally were similar from the six laboratories except where hard water increased the values for silver. They ranged from 0.6 to 55.0 $\mu g\, l^{-1}$ for silver, and from 158 to 720 $\mu g\, l^{-1}$ for endosulfan (Table 2). ABC Laboratories had hard well water, which explains their much higher EC50

Table 2. *Daphnia magna* 48-h EC50 values for endosulfan and silver

Laboratory	Silver ($\mu g\, l^{-1}$) 48-h EC50 (CI)	Endosulfan ($\mu g\, l^{-1}$) 48-h EC50 (CI)
ABC*		390 (310–490)
	48.0 (42–54)	630 (550–720)
	55.0 (52–59)	720 (580–900)
DuPont†	8.4 (7.5–9.2)	372 (325–432)
	14.9 (14.3–15.5)	328 (293–371)
ERL-Corvallis	1.1 (0.9–1.5)	343 (263–447)
	0.6 (0.5–0.8)	271 (226–325)
ERL-Duluth	0.63 (0.4–1.5)	218 (91–572)
	0.66 (0.5–1.3)	282 (245–328)
SRI	0.9 (0.8–1.1)	266 (241–296)
	1.03 (0.9–1.2)	158 (137–186)
		378 (220–645)‡
WAPORA	2.9 (2.3–3.6)	740 (521–1051)‡
		280 (217–361)

* ABC Laboratories had very hard well water, which explains their much higher EC50 values for silver.

†DuPont water had an intermediate hardness, giving somewhat higher EC50 values.

‡ LC50 value.

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